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Amphiphysin 1 Binds the Cyclin-dependent Kinase (cdk) 5 Regulatory Subunit p35 and Is Phosphorylated by cdk5 and cdc2*

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Amphiphysin 1 is a phosphoprotein expressed at high levels in neurons, where it participates in synaptic vesicle endocytosis and neurite outgrowth. It is a substrate for cyclin-dependent kinase (cdk) 5, a member of the cyclin-dependent protein kinase family, which has been functionally linked to neuronal migration and neurite outgrowth via its action on the actin cytoskeleton. The yeast homologue of amphiphysin, Rvs167, functions in endocytosis and actin dynamics, is phosphorylated by the cdk5 homologue Pho85, and binds the Pho85 regulatory subunit Pcl2. We show here that amphiphysin 1 interacts with the cdk5-activating subunit p35 and that this interaction is mediated by the conserved NH_2-terminal region of amphiphysin. Amphiphysin 1 colocalizes with p35 in the growth cones of neurons and at actin-rich peripheral lamellipodia in transfected fibroblasts. Amphiphysin is phosphorylated by cdk5 in a region including serines 272, 276, and 285. Amphiphysin 1 is also phosphorylated by the cdc2/cyclin B kinase complex in the same region and undergoes mitotic phosphorylation in dividing cells. These data indicate that phosphorylation by members of the cyclin-dependent kinase family is a conserved property of amphiphysin and suggest that this phosphorylation may play an important physiological role both in mitosis and in differentiated cells.

Amphiphysin 1 and 2 are SH3 domain-containing proteins concentrated in nerve terminals of mature neurons (1, 2). They belong to a protein family conserved from yeast to humans, whose members play pleiotropic roles in endocytosis, actin function, and regulation of growth control. Studies of the yeast amphiphysin homologue Rvs167 have shown that this protein is phosphorylated by the Pho85 kinase and binds Pcl2, an activator of this kinase (3). The Pho85 kinase is a member of the cyclin-dependent kinase family and functions in cellular growth control as well as endocytosis and actin function. The homologue of the Pho85 kinase in mammalian cells is cdk5[1] (4, 5). Its two activators, p35 and p39 (6–8), which represent functional homologues of Pcl2, are expressed only in neurons and developing muscle cells (6, 7, 9). Consistent with these homologies, amphiphysin 1 is a substrate for cdk5 and is a component of a high molecular weight complex in brain that also contains cdk5/p35 (10). In addition to its role in neurons, amphiphysin is likely to function outside the nervous system because it is expressed in other cell types (11, 12). In this study we have further characterized the relationship between amphiphysin 1 and the cdk5/p35 kinase complex. We also show that amphiphysin 1 is phosphorylated by cdc2, another cyclin-dependent kinase family member, and undergoes mitotic phosphorylation.

MATERIALS AND METHODS

Antibodies—Amphiphysin polyclonal antibodies CD5 and CD9 and monoclonal antibodies have been described previously (11, 13). p35 antibodies were obtained from Santa Cruz Biologicals (antibody C-19) or were generated as described previously (14). Anti-cdc2 antibody was obtained from Transduction Laboratories, and anti-cdk5 antibody was obtained from Upstate Biotechnology. Nonimmune control rabbit IgG was obtained from Sigma.

Glutathione S-Transferase (GST) Fusion Proteins—cDNAs encoding sequences of p35, p25, cdk5, or amphiphysin 1 were subcloned into either pGEX2T, pGEX4T, or pGEX6P (Pharmacia) as described previously (15, 16). Fusion proteins were prepared according to the manufacturer's instructions. Baculovirus-expressed human GST-cdc2/cyclin B1 with activating T14A and Y15F mutations as described previously (17) was a kind gift of Dr. Graham Warren (Yale University, New Haven, CT).

In Vitro Binding Assay—p35 was [^{35}S]methionine-radiolabeled using a coupled *in vitro* transcription/translation kit (Promega). 5 μl of the total reaction mixture was combined with 10 μg of recombinant GST protein fused to amino acids 1–161, 1–246, 1–306, 262–435, 411–581, and 545–695 of the human amphiphysin 1 sequence in 1 ml of buffer A (150 mM NaCl, 50 mM Tris-HCL, 5 mM EDTA, and 1% Triton X-100). These reactions were incubated for 1 h at room temperature, combined with 50 μl of 50% slurry of glutathione-Sepharose in buffer A, incubated for 30 min at room temperature, washed four times with 1 ml of buffer A, separated by SDS-PAGE, and dried to Whatman filter paper. Radioactive protein bands were detected by fluorography.

In Vitro Kinase Assays—Kinase assays were performed as described previously (18), with the following modifications: 1 μM purified histone H1 (Roche) or amphiphysin fusion protein cleaved from the GST tag according to the manufacturer's instructions was included in a total reaction volume of 50 μl containing either 10 μg of purified GST-cdk5 and 10 μg of purified GST-p25, anti-p35 immunoprecipitate from rat brain extract, or 1 μg of GST-tagged human cyclin B1/cdc2. [γ-^{32}P]ATP was included at a final specific activity of 1–10 Ci/mol in a total concentration of 200 μM. The reaction mixtures were incubated at room temperature for 30 min, and the reaction was stopped by adding sample buffer and boiling for 1 min. The proteins were separated by SDS-PAGE, the gels were dried to Whatman filter paper, and radioactive

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[1] The abbreviations used are: cdk, cyclin-dependent kinase; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; CHO, Chinese hamster ovary.

protein bands were detected by autoradiography. Radioactive bands were quantitated on a STORM 860 PhosphorImager (Molecular Dynamics) or by scanning on a Gel Doc 2000 densitometer (Bio-Rad). For SDS-PAGE mobility assays, 1 μM amphiphysin 1 or mutant 2 was incubated with 200 μM ATP in the presence or absence of 1 μg of GST cyclin B1/cdc2 overnight at room temperature in a total volume of 50 μl. Proteins were then separated on 6% SDS-PAGE gels and probed by Western blot with monoclonal amphiphysin 1 antibody.

Tryptic Digestion and Identification of Phosphorylated Peptides—50 μg of recombinant amphiphysin 1 was ^{32}P-radiolabeled *in vitro* using p35 immunoprecipitates from rat brain as described above. The reaction products were separated by SDS-PAGE and stained with Coomassie Blue, and the amphiphysin band was excised. In conjunction with the Keck Foundation Biotechnology Resource Laboratory at Yale University, this sample was trypsin-digested, and peptides were eluted from the gel and separated by HPLC as described previously (19, 20). Radioactive fractions were detected by Cerenkov counting, and constituent peptides were determined by matrix assisted-laser desorption ionization mass spectrometry as described previously (21) and Edman sequencing on a Procise cLC instrument (PerkinElmer Life Sciences) as per the manufacturer's protocols.

Mutagenesis—Constructs containing the mutations T260L, S262A, S268A (Mut 1), S272A, S276A, S285A (Mut 2), T260L, S262A, S268A, S272A, S276A, and S285A (Mut 3) in the human amphiphysin 1 sequence were generated by polymerase chain reaction, digested with restriction enzyme *Dpn*I, and transformed into competent bacteria as described previously (22).

Mitotic Synchronization of CHO Cells—CHO cells stably transfected with amphiphysin 1 were synchronized with nocodazole as described previously (23). Mitotic cells were harvested and homogenized or allowed to progress to G_1 phase before harvesting. Proteins were analyzed by SDS-PAGE and Western blotting.

Cell Culture and Transfections—Culture of primary rat cortical neurons was performed as described previously (24, 25). An amphiphysin stable cell line was generated by transfecting Chinese hamster ovary cells maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum with human amphiphysin cDNA in pRc/RSV vector (Invitrogen). Transfected cells were selected by neomycin resistance, and serial dilutions were performed to obtain single clones. Cos7 cells were maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum. Transient transfections were performed using p35 or amphiphysin 1 in vector pcDNA3 and LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions.

Miscellaneous—Immunoprecipitation, immunostaining, and SDS-PAGE were performed as described previously (15). Triton X-100 extracts of whole rat brain were prepared as described previously (15).

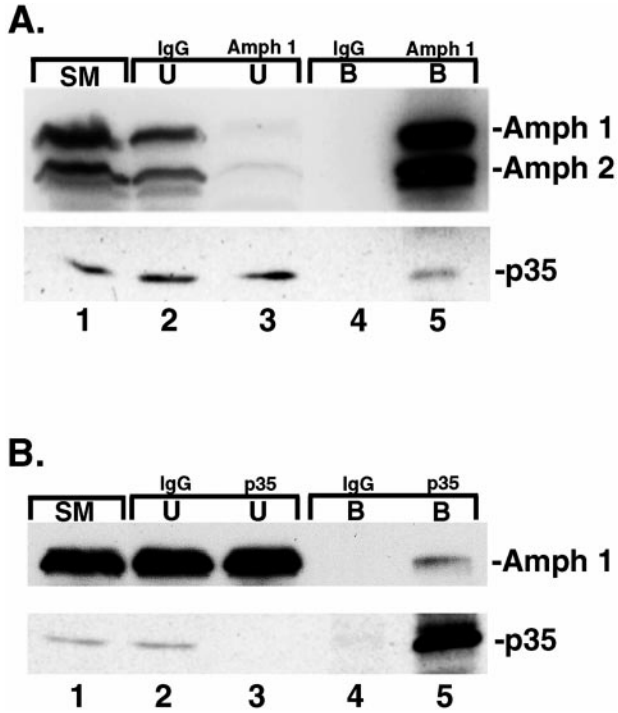

FIG. 1. **Amphiphysin and p35 exist together in a complex in rat brain.** *A*, immunoprecipitate from rat brain using control anti-cdc2 (*lane 4*) or anti-amphiphysin monoclonal 8 (*lane 5*) antibodies Western blotted with anti-amphiphysin 1 and 2 CD9 (*top panel*) and p35 C-19 (*bottom panel*) antibodies indicates that p35 coprecipitates with amphiphysin. One-tenth of the starting material used for immunoprecipitation is loaded in *lane 1*, and unbound fractions from control and amphiphysin immunoprecipitation are loaded in *lanes 2* and *3*, respectively. *B*, immunoprecipitate from Triton X-100 extract of total rat brain homogenate using nonspecific rabbit immunoglobulin (*lane 4*) or anti-p35 antibodies (14) (*lane 5*) Western blotted with anti-amphiphysin 1 monoclonal antibody 3 (*top panel*) or anti-p35 C-19 antibodies (*bottom panel*) shows that amphiphysin 1 coprecipitates with p35 from rat brain. One-tenth of starting material is loaded in *lane 1*, and unbound fractions from control and p35 immunoprecipitation are loaded in *lanes 2* and *3*, respectively.

RESULTS

Amphiphysin and p35 Interact in Brain and in Transfected Cells—Recently, amphiphysin has been identified as a substrate and interactor of the cdk5 kinase complex (10). To further investigate this interaction, we performed immunoprecipitation experiments from rat brain extracts. Anti-amphiphysin antibodies purified the amphiphysin 1–2 heterodimer and coprecipitated p35, the activating subunit of the cdk5 kinase complex. Likewise, anti-p35 antibodies coprecipitated amphiphysin (Fig. 1). cdk5 was not detected in anti-amphiphysin immunoprecipitates, indicating that amphiphysin does not interact directly with the kinase and that a ternary complex of amphiphysin, p35, and cdk5 is not present at significant levels (data not shown).

We probed the interaction of amphiphysin with p35 by reconstituting it in a transfected cell system. To this end, a CHO cell line stably transfected with amphiphysin 1 was transiently transfected with either p35 or empty control vector. As shown in Fig. 2, immunoprecipitation with antibodies directed against either amphiphysin or p35 precipitated a complex containing both proteins. cdk5 was endogenously expressed in this cell line and could be immunoprecipitated by anti-p35 antibodies (Fig. 2*B*, *bottom panel*). However, in agreement with our observation in brain extracts, amphiphysin immunoprecipitates did not contain cdk5.

Amphiphysin 1 and p35 Colocalize in Vivo—Based on the results of coprecipitation experiments, we investigated whether p35 and amphiphysin colocalize in cells. Both p35/cdk5 and amphiphysin have been implicated in neurite outgrowth and neuronal migration (14, 26–29). As shown by Fig. 3, *A* and *B*, endogenous amphiphysin 1 and p35 colocalize in the growth cones of rat cortical neurons in culture, consistent with a possible role for their interaction in growth cone navigation.

Similar observations were made in Cos7 cells cotransfected with amphiphysin 1 and p35 cDNAs. In these cells, a large fraction of both proteins had a diffuse cytosolic distribution. In addition, pools of amphiphysin 1 and p35 were colocalized in ruffles and lamellipodia at the cell periphery (Fig. 3, *C* and *D*). These structures were also positive for filamentous actin, as demonstrated by phalloidin immunostaining. These findings are consistent with previous observations that both amphiphysin and the p35/cdk5 complex are present at actin-rich lammellipodial structures in neuronal growth cones (14, 28).

p35 Binds the NH_2-terminal Region of Amphiphysin 1—To map the domain of amphiphysin 1 that binds to p35, we radiolabeled p35 by coupled *in vitro* transcription/translation and performed *in vitro* binding experiments with glutathione *S*-transferase-tagged fragments of amphiphysin 1. As shown in Fig. 4, the first 306 amino acids of amphiphysin 1 were sufficient to retain *in vitro* translated p35 on glutathione-Sepharose beads. These data confirm the interaction of amphiphysin 1 with p35 and demonstrate that the binding site for p35 resides in the NH_2-terminal domain of the molecule.

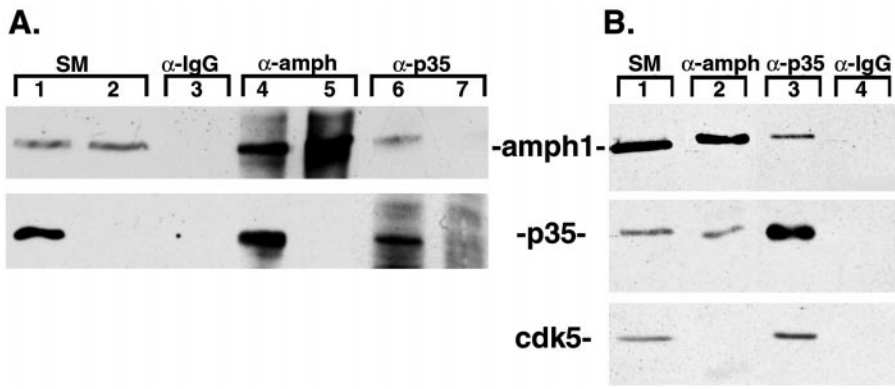

FIG. 2. **Amphiphysin and p35 interact in transfected cells in a complex that does not include cdk5.** *A*, CHO cells stably transfected with amphiphysin 1 were mock-transfected with empty vector (*lanes 2, 5,* and *7*) or vector containing p35 (*lanes 1, 3, 4,* and *6*). Triton X-100 extracts of cells were prepared, and starting material (*lanes 1* and *2*) or immunoprecipitates using control IgG (*lane 3*), anti-amphiphysin monoclonal 3 (*lanes 4* and *5*), and anti-p35 (14) (*lanes 6* and *7*) antibodies were probed by Western blot with amphiphysin monoclonal antibody 3 (*top panel*) or p35 C-19 antibody (*bottom panel*). Amphiphysin was detected in p35 precipitates, and p35 was detected in amphiphysin precipitates. *B*, immunoprecipitates from Triton X-100 extracts of CHO cells stably transfected with amphiphysin and transiently transfected with p35 were prepared using amphiphysin polyclonal antibody CD5 (*lane 2*), p35 polyclonal antibody (14) (*lane 3*), or control IgG (*lane 4*). These immunoprecipitates and one tenth of the starting material (Triton-100 extract, SM, *lane 1*) were probed by Western blot with amphiphysin monoclonal antibody 3 (*top panel*), p35 C-19 (*middle panel*), or cdk5 (*bottom panel*) antibodies. p35 coprecipitated cdk5, but amphiphysin did not, indicating that amphiphysin does not bind directly to cdk5 and that a ternary complex was not present at detectable levels.

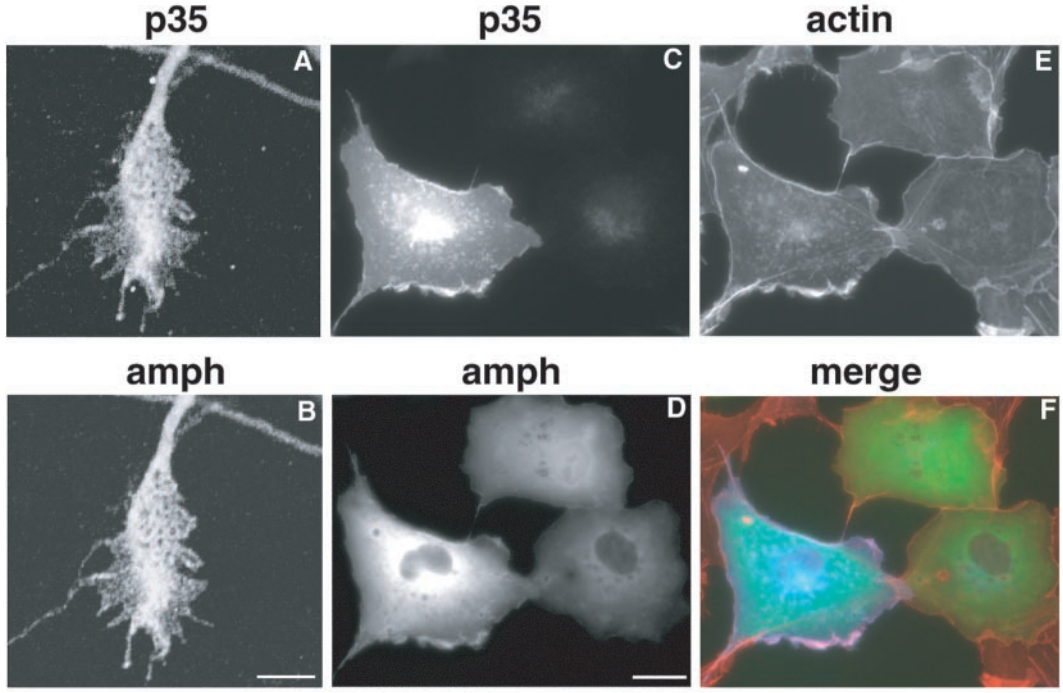

FIG. 3. **Amphiphysin and p35 colocalize in growth cones of neurons in culture and at actin-rich structures in transfected fibroblasts.** *A* and *B*, P1 rat cortical neurons were fixed and immunostained with anti-p35 (14) (*A*) or anti-amphiphysin monoclonal 3 (*B*) antibodies and visualized by confocal scanning microscopy. *Scale bar* in *B* represents 2 μM. *C—F*, light micrographs of Cos7 fibroblasts transiently transfected with amphiphysin 1 and p35 and then fixed and immunolabeled with anti-p35 C-19 polyclonal antibody (*C*), amphiphysin monoclonal antibody 3 (*D*), and rhodamine phalloidin (*E*). *F* shows a pseudocolored image with amphiphysin in *green*, p35 in *blue,* and actin in *red*. In cells coexpressing amphiphysin and p35, a pool of amphiphysin is found at actin-rich structures near the cell periphery. *Scale bar* in *D* represents 10 μM.

Amphiphysin 1 is Phosphorylated by cdk5 between Amino Acids 254 and 320—We next investigated cdk5 phosphorylation of amphiphysin 1. To this end, we prepared immunoprecipitates of the p35/cdk5 complex from rat brain using anti-p35 antibodies. These immunoprecipitates were then used in *in vitro* kinase reactions with recombinant amphiphysin 1 or histone H1, a known cdk5 substrate. As shown in Fig. 5, *lanes a–d*, p35 immunoprecipitates, but not control IgG immunoprecipitates, incorporated radiolabeled phosphate into both histone H1 and amphiphysin 1. *In vitro* kinase reactions performed with bacterially expressed recombinant p35/cdk5 instead of immunoprecipitates gave similar results, indicating that amphiphysin 1 is phosphorylated by cdk5 and not by a contaminating kinase from brain extracts (data not shown).

To determine what region of amphiphysin 1 is phosphorylated by cdk5, recombinant peptides corresponding to overlapping regions of the protein were tested in *in vitro* kinase reactions. This strategy demonstrated the presence of a major phosphorylation site or sites selectively in amphiphysin 1 fragments comprising amino acids 1–306 and 262–435 (Fig. 5). Strikingly, the region of overlap between these two peptide fragments, but not other regions of the two fragments, contains

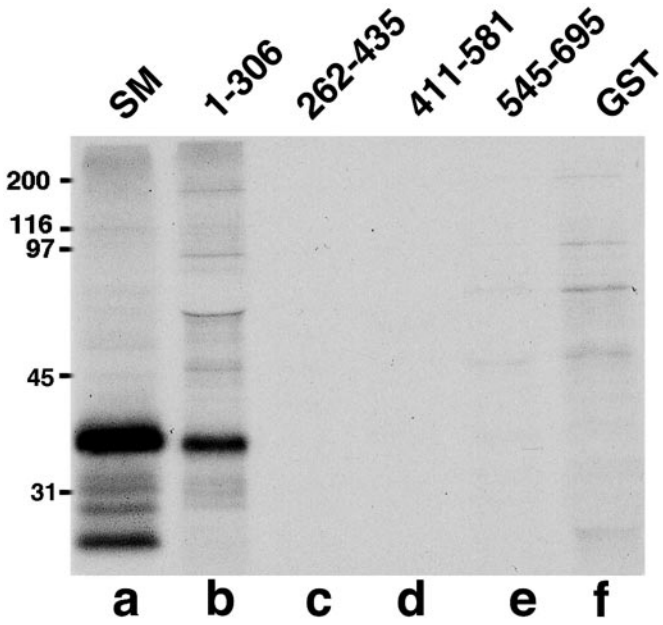

FIG. 4. **The NH_2 terminus of amphiphysin binds p35 *in vitro*.** GST-tagged overlapping fragments of amphiphysin 1 corresponding to the indicated *amino acid numbers* were incubated with radiolabeled *in vitro* translated p35 and then isolated on glutathione-Sepharose beads. *Lane a* shows the starting material for the binding reaction. The NH_2-terminal 306 amino acids of amphiphysin were able to affinity-purify p35 (*lane b*), whereas neither the remaining COOH-terminal fragments (*lanes c–e*) nor GST alone (*lane f*) bound p35.

several potential consensus phosphorylation motifs for cdk5, as indicated in Fig. 6*A*.

To confirm that the region between amino acids 262–306 contained the phosphorylation site for cdk5, tryptic digestion of *in vitro* ^{32}P-phosphorylated amphiphysin 1 was performed, and the resulting peptide fragments were separated by HPLC chromatography. Chromatographic fractions were then assayed for radioactivity, and the radioactive peptides were identified by matrix-assisted laser desorption mass spectroscopy and subsequent microsequence analysis. By this method, we identified a phosphorylated peptide corresponding to amino acids 260–292 (*arrowheads* in Fig. 6*A*), in agreement with the results shown in Fig. 5.

We mapped which amino acid residues of amphiphysin 1 within this region are targets for phosphorylation by cdk5 by generating full-length amphiphysin 1 constructs in which threonine 260, serine 262, serine 268, serine 272, serine 276, and serine 285 were mutated to nonphosphorylatable residues. These recombinant proteins were then tested in *in vitro* kinase reactions using the p35/cdk5 complex immunoprecipitated from rat brain. This strategy demonstrated that mutation of threonine 260, serine 262, and serine 268 (Fig. 6, *Mut 1*) did not affect amphiphysin phosphorylation, whereas constructs harboring mutations of serine 272, serine 276, and serine 285 (Fig. 6, *Mut 2* and *3*) showed reduced phosphorylation. The cdk5 phosphorylation site or sites in amphiphysin therefore reside in one or more of these three amino acids.

Amphiphysin Is a Substrate for the cdc2 Kinase and Is Phosphorylated during Mitosis—Members of the cyclin-dependent family of kinases require similar sequence motifs in their respective substrates (30). Two other proteins implicated in clathrin-mediated endocytosis and actin function, epsin and eps15, are substrates for cyclin-dependent kinases in mitotic cells (23, 31). Whereas amphiphysin 1 is expressed at high concentration in neurons, it is also expressed at lower levels in other cells, including dividing cells (11, 12). In addition, it is overexpressed in some breast tumors and breast cancer cell lines and was identified as a paraneoplastic syndrome autoantigen in patients with breast cancer (2, 11, 32). These considerations prompted us to investigate whether amphiphysin undergoes mitotic phosphorylation.

Based on substrate sequence preference similarities among cyclin-dependent kinases, we tested whether amphiphysin 1 is phosphorylated *in vitro* by the mitotic cyclin-dependent kinase cdc2/cyclin B1. To this end, we used a purified recombinant cdc2/cyclin B1 complex in which cdc2 harbors an activating mutation (17). As shown in Fig. 7*A*, full-length amphiphysin 1 was phosphorylated by this complex. Using amphiphysin fragments, we mapped the major cdc2/cyclin B1 phosphorylation site(s) to the region between amino acids 262 and 306, *i.e.* the same region that is phosphorylated by cdk5 (Fig. 7*A*). Both cdc2/cyclin B1 and cdk5 showed reduced phosphorylation of the amphiphysin mutant (Mut 2) harboring serine to alanine substitutions at positions 272, 276, and 285, indicating that amphiphysin is phosphorylated at these residues by both of these kinases (Fig. 7*B*).

We next tested phosphorylation of amphiphysin in mitotic cells. CHO cells stably transfected with amphiphysin 1 were mitotically synchronized using nocodazole, and the electrophoretic mobility of amphiphysin 1 was analyzed by Western blotting in the mitotic and G_1 stages. As shown by Fig. 7*C*, amphiphysin 1 undergoes a mobility shift in mitotic cells. This slower mobility, which has been observed for several other mitotic phosphoproteins, could be reversed by alkaline phosphatase treatment of the mitotic extract, as expected for a shift due to phosphorylation (Fig. 7*C*, *lane 2*). Interestingly, cdc2/cyclin B1 could induce an electrophoretic shift in recombinant amphiphysin 1 in *in vitro* kinase reactions (Fig. 7*D*). This shift was reduced in the Mut 2 amphiphysin construct harboring serine to alanine mutations at residues 272, 276, and 285. Note, however, that even the Mut 2 variant of amphiphysin runs slightly slower than wild type amphiphysin in SDS-PAGE after the *in vitro* kinase reaction with cdc2/cyclin B1 complex. This observation suggests that under these *in vitro* conditions, cdc2 can phosphorylate additional sites.

DISCUSSION

In this study we report evidence for a functional link between amphiphysin 1 and the cdk5 kinase complex. We have demonstrated the *in vivo* occurrence of an interaction between amphiphysin and the cdk5 regulatory subunit p35 by immunoprecipitation and immunofluorescence. This interaction is analogous to the Pcl2-Rvs167 interaction in yeast. We have also shown that p35 binds to the evolutionary conserved NH_2-terminal region of amphiphysin 1, in agreement with the reported binding of Pcl2 to the NH_2-terminal moiety of Rvs167 (3). Thus, our findings point to a further similarity between the mammalian p35/cdk5/amphiphysin 1 and the yeast Pcl2/Pho85/Rvs167 protein networks. We also mapped the main cdk5 phosphorylation site to a small fragment containing three serine residues in the central region of amphiphysin 1, just upstream of the binding sites for clathrin and the clathrin adaptor AP-2, although phosphorylation of this site did not appear to affect AP-2 or clathrin binding.[2] Because Rvs167, like several other members of the amphiphysin family, does not contain recognizable clathrin or AP-2 binding sites (33–35), it is not unexpected that cdk5 phosphorylation may not represent a conserved mechanism to regulate these interactions.

Studies of p35/cdk5 have revealed a key role of this complex in the regulation of actin function at the leading edge of neu-

[2] S. R. Floyd, E. B. Porro, V. I. Slepnev, G.-C. Ochoa, L.-H. Tsai, and P. De Camilli, unpublished observations.

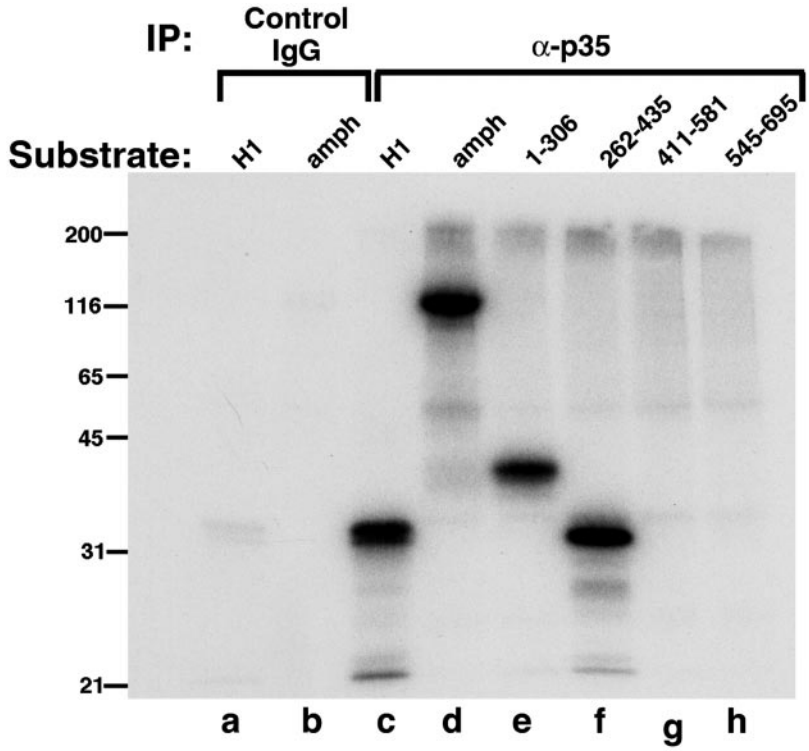

FIG. 5. **Amphiphysin is phosphorylated by cdk5 between amino acids 254 and 320.** Immunoprecipitates from rat brain extract using nonspecific rabbit IgG (*lanes a* and *b*) or anti-p35 antibodies (*lanes c–h*) were combined with purified recombinant histone H1 or recombinant amphiphysin 1 peptides as indicated. These mixtures were combined with radiolabeled ATP, incubated, separated by SDS-PAGE, and exposed to film. Histone H1 (*lane c*), full-length amphiphysin 1 (*lane d*), and fragments corresponding to amino acids 1–306 (*lane e*) and 262–435 (*lane f*) show incorporation of radiolabeled phosphate.

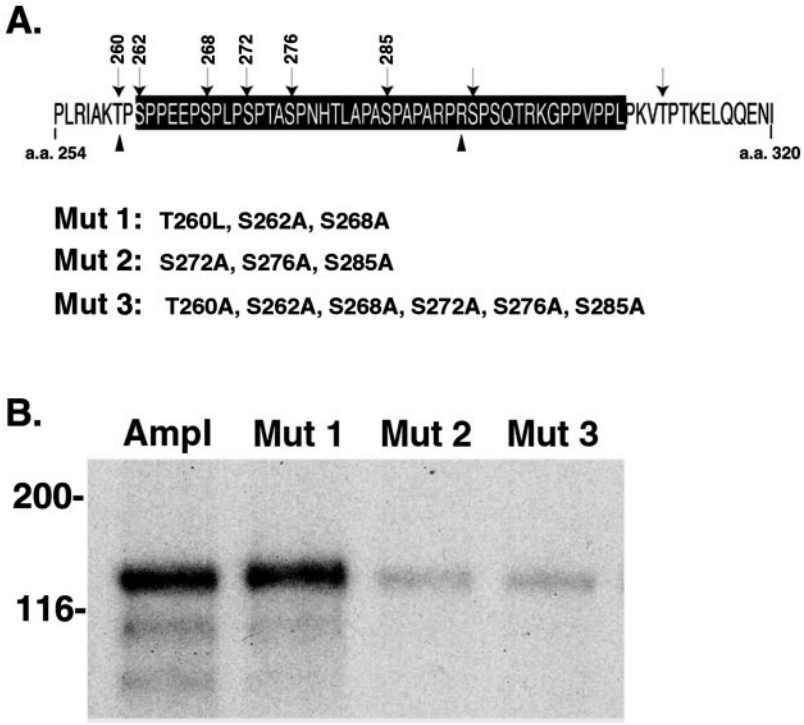

FIG. 6. **Amphiphysin 1 is phosphorylated at amino acids 272, 276, and 285 *in vitro*.** *A*, amino acid sequence of the phosphorylated region from Fig. 5; *arrows* indicate consensus phosphorylation sites for cdk5. The boxed region indicates the overlap of peptides from Fig. 5, lanes *e* and *f*. *Arrowheads* denote the peptide sequence identified by matrix assisted-laser desorption ionization mass spectrometry and Edman sequencing of tryptic digest of amphiphysin *in vitro* kinase reaction using p35 immunoprecipitate from rat brain. GST fusion proteins corresponding to full-length amphiphysin 1 with mutations at the indicated amino acid positions were prepared using polymerase chain reaction-based mutagenesis. *B*, equal molar amounts of these proteins were then tested in *in vitro* kinase assays using immunoprecipitated p35/cdk5 for incorporation of radiolabeled phosphate. Mutants 2 and 3 (*Mut 2* and *Mut 3*) showed a significant decrease in phosphate incorporation. These two constructs share mutations in serine 272, 276, and 285, indicating that these residues are phosphorylated by cdk5.

ronal processes with important functional implications for neuronal migration and growth cone navigation (14, 26, 36). Likewise, independent studies have suggested a role of amphiphysin and its binding partners in growth cone dynamics (28). Amphiphysin has also been implicated in endocytosis, a process for which a key role of actin is emerging (37, 38). Although a function of cdk5 phosphorylation in endocytosis has not been reported, in yeast, Pho85, Plc2, and Rvs167 mutations have strikingly similar effects on both actin function and endocytosis (3). We suggest therefore that amphiphysin, p35, and cdk5 may be interrelated in their physiological functions *in vivo*. Our demonstration that amphiphysin 1 and p35 colocalize with each other and with actin in lamellipodia of transfected cells supports this possibility. We note that an interaction between amphiphysin 2 and the cAbl kinase has been reported (39). cAbl has recently been shown to phosphorylate and activate cdk5 and to bind cdk5 through the bridging protein cables (40). In addition, cAbl is known to have regulatory actions on the actin cytoskeleton and participate in neuronal development (41, 42).

Finally, we have shown that amphiphysin 1 undergoes mitotic phosphorylation. The same region that is a target for cdk5 phosphorylation can also be phosphorylated by the mitotic

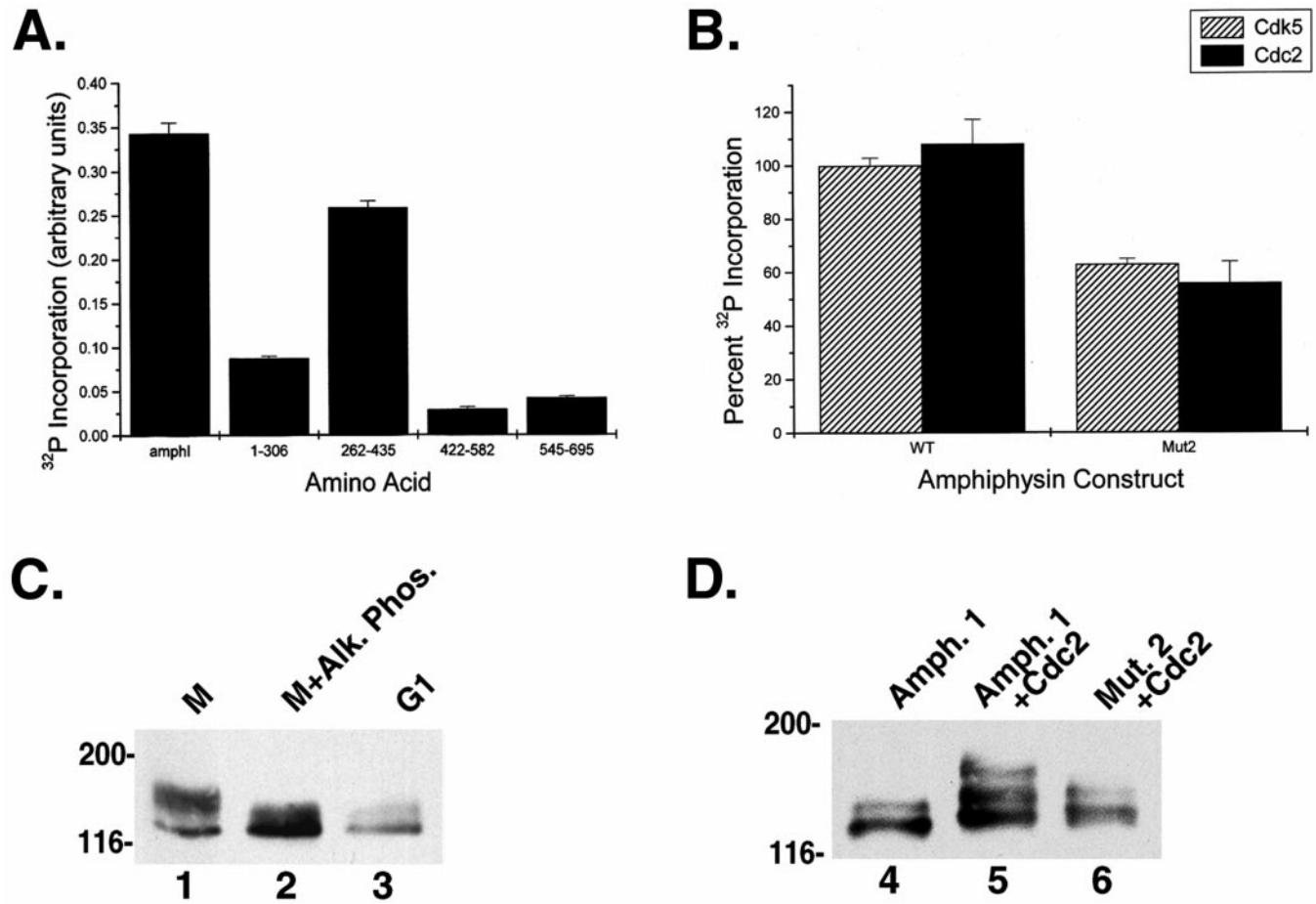

FIG. 7. **Amphiphysin is phosphorylated by cdc2/cyclin B1 at amino acids 272, 276, and 285 *in vitro* and undergoes mitotic phosphorylation.** *A*, purified recombinant human cdc2/cylcin B1 was combined with equal molar amounts of purified recombinant amphiphysin 1 peptides and radiolabeled ATP as indicated. Radioactive bands were quantitated by densitometry, and the results shown are the mean of four experiments. *Error bars* are the standard error of the mean. Full-length amphiphysin 1 and fragments 1–306 and 262–435 show incorporation of radioactive phosphate. *B*, full-length wild type (*WT*) or mutant 2 (*Mut2*) amphiphysin constructs as described in the Fig. 6 legend were tested in *in vitro* kinase reactions using recombinant cdc2/cyclin B1 (■) or p35/cdk5 (▨). Radioactive bands were quantitated by phophorimaging, and the results shown are the mean of three experiments. *Error bars* are the standard error of the mean. Mutation of serine 272, 276, and 285 to alanine reduced incorporation of radiolabeled phosphate by both cdc2 and cdk5. *C*, CHO cells stably transfected with amphiphysin 1 were synchronized with nocodazole and separated into mitotic and G_1-phase pools. Triton X-100 extracts of these pools were probed by Western blotting using anti-amphiphysin 1 monoclonal 3 antibody. Amphiphysin expressed in mitotic cells (*lane 1*) migrates more slowly on SDS-PAGE than amphiphysin in G_1-phase cells (*lane 3*). Treatment of the mitotic cell extract with alkaline phosphatase abolished this migration shift (*lane 2*). *D*, recombinant wild type amphiphysin 1 (*lanes 4* and *5*) or mutant 2 (*lane 6*) was incubated with ATP alone (*lane 4*) or recombinant cyclin B1/cdc2 and ATP (*lanes 5* and *6*) and then probed by Western blot with an amphiphysin monoclonal antibody. Incubation of wild type amphiphysin with cdc2 and ATP resulted in a migration shift on SDS-PAGE. This shift was reduced by serine to alanine mutations at amino acids 272, 276, and 285.

cdc2/cyclin B1 complex. Thus, phosphorylation of amphiphysin 1 in this region may have a similar conserved function in both neurons and dividing cells. One of the critical events that correlates with mitosis is the dramatic rearrangement of the peripheral cytoplasm that results in the partial dissociation of cells from the substratum. Cyclin-dependent kinase phosphorylation of amphiphysin may help to produce local changes in the actin cytoskeleton that are crucial for the dynamic properties of dividing cells, neuronal growth cones, the leading edge of migrating cells, and the function of the mature synapse.

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